AD-A173 061 CELLULAR EFFECTS OF PERFLUORINATED FATTY ACIDS(U) 1/1
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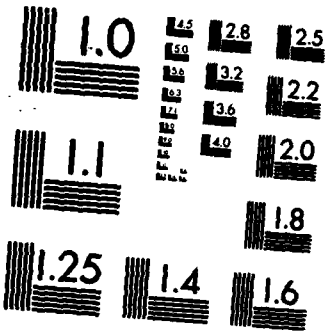

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The compound perfluoro-n-decanoic acid (PFDA) was exposed to three tissue culture cell lines, PTK2 (kidney), BRL (buffalo rat liver) and K9 (clone 9, rat liver) in concentrations up to 200 ug/ml for 24 hr. time periods. The technique of fluorescence recovery after photobleaching (FRAP) was used to examine cell membrane fluidity, specifically with respect to the membrane lipids. Results demonstrated that PFDA affects the fluidity of both liver lines but not the kidney line. Furthermore, differential sensitivity was observed between the responsiveness of the two liver lines. (BRL was more sensitive than K9.) The increased PFDA-induced membrane fluidity was transient: recovery occurred by 48 hr following removal of the PFDA. The FRAP technique appears useful in screening agents for membrane effects as well as elucidating mechanisms of agent action.

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INTRODUCTION

In recent years, research has indicated that the cellular membrane is an important target in the cytotoxic action of a variety of environmentally and industrially important chemical agents (1,2,3,4,5,6). It is well known that the structural and functional integrity of the cell membrane is crucial for the maintenance of normal cell function and viability. This is true for all cell systems. In particular, previous in vivo and in vitro studies on the similarities of the toxic effects of perfluoro-n-decanoic acid (PFDA) and 2,3,7,8-tetrachlorodibenzodioxin (TCDD) suggested that their toxicities may be due to an ability of these chemicals to interfere with normal structure and/or function of biological membranes (1,2,3). Changes in red blood cell fragilities were induced by PFDA. Both in vivo and in vitro, PFDA and TCDD appeared to interfere with fatty acid metabolism leading to an increase in unsaturation. Furthermore, Andersen et al. (2) proposed that such an increase in cellular unsaturated fatty acids may lead to excessive membrane fluidity (as indicated by induced changes in red blood cell fragility) and consequent impaired membrane function. In order to investigate this target site problem more closely and, in particular, to elucidate possible mechanisms of action of PFDA (which is a major ingredient in fire-fighting materials), we have employed the technique of "fluorescence recovery after photobleaching," FRAP. This sensitive method for the determination of cell membrane fluidity could be very useful in delineating the extent and nature of the effect of toxic species on the cell membrane.

MATERIALS AND METHODS

Tissue Culture

Three established cell lines from the American Type Culture Collection (ATCC) were employed: PTK_2 cells (marsupial, Potorous tridactylis kidney cells), BRL (buffalo rat liver cells, CRL 1442) and clone 9 (abbreviated K9, normal rat liver cell, CRL 1439). The two liver cell lines have been shown to exhibit certain biochemical properties characteristic of differentiated liver cells (7). The cells were grown in Falcon T25 tissue culture flasks and also in Rose chambers and incubated in an incubator of humidified environment of 5% CO_2:95% air at 37°C.

The PTK_2 cells were maintained in Eagle's MEM with 10% fetal calf serum and subcultured with 0.125% Viokase. The BRL cells were cultivated in Ham's F12 medium with 5% fetal calf serum and subcultured with CTC (collagenase, trypsin and chick serum) in Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+}. The clone 9 cells were grown in Ham's F12 medium with .25% trypsin. The media of all cells were changed every 2-3 days and subcultured once a week at a density of 4-6 x 10^5 cells/ml. For FRAP and SEM studies, cells were inoculated into Rose chambers at a density of 2 x 10^4 cells/ml, and experiments were carried out with cultures that were 1-7 days old.

SEM

Cells of the BRL line were grown in monolayer culture in Rose chambers and then treated with the maximum effective non-toxic dose of PFDA (100 ug/ml) for 24 hrs. Both the treated and control samples were fixed with 3% gluteraldehyde in Millonig's phosphate buffer and culture medium for 30 min, washed 3 times, 15 min each, in Millonig's phosphate buffer, pH 7.4. The fixed specimens were then dehydrated through a series of 30-100% EtOH and

then 30-100% Freon 113/EtOH, 5 min incubation for each concentration. Critical point drying with Freon 13 was performed with Omar critical point dryer, then gold coated with Hummer II. The samples were observed under the Hitachi S500 scanning electron microscope at 10 kV, 45° tilt and aperture opening diameter of 300 um.

Cell Surface Labeling

The Rose chambers were disassembled, and the coverslip containing the cells was placed with the cell side down on a glass slide onto which had previously been placed several drops of media held between two parallel beads of vaseline. The vaseline sealed the edges of the coverslips, but not the ends, creating a perfusion chamber. Media were perfused continuously through the chamber with the aid of a pipet at each end. Cells were labeled with a fluorescently labeled lipid membrane probe, 10 ug/ml 5(N-hexadecanoyl) aminofluorescein, through incubation in the above solutions for 10 min and subsequently washed 3 times, 5 min each, with Hanks BSS. The perfusion chamber was then sealed with a hot mixture of vaseline:parafin:lanolin, and photobleaching measurements were made within 2 hrs.

FRAP

A Liconix HeCd laser was used as a source of monochromatic beam of wavelength 441 nm to excite fluorescent emission when attenuated and bleaching of fluorescent probe when unattenuated. An array of optical processing equipment was employed to obtain a laser microspot with a Gaussian profile and a diameter of ~1.4 um focused onto the optical plane of a Zeiss standard RA microscope. The intensity of emitted fluorescence was

monitored through an EMI #9862B/100 photomultiplier tube which transmitted the signals to a Tracor Northern TN-1710 multichannel analyzer.

The initial intensity of fluorescence was monitored, expressed as counts/second (cps), and recorded by the multichannel analyzer. The fluorophore at the cell surface was bleached with the unattenuated laser beam for a duration of 0.1 sec to achieve optimal bleaching. The recovery of fluorescence intensity with time at the microspot was subsequently monitored with the attenuated beam until a plateau stage was attained. The data curve was then stored onto computer disks for analysis. Following the bleaching pulse, the neighboring mobile fluorophores migrated to the bleached spot resulting in a gradual increase of fluorescent intensity with time. The fluidity of the membrane molecules was measured and expressed as a diffusion constant in units of 10^{-9} $cm^2 sec^{-1}$ according to the following equations:

$$D = \frac{w_o^2}{4t_{1/2}} \gamma D$$

t = time (sec)
γ = constant
w_o = $(\frac{1}{e^2})$ radius of laser beam

PFDA Treatment

PFDA was dissolved in DMSO to yield a stock solution of 100 mg/ml, and solutions of 10 to 1,000 ug/ml PFDA in respective culture media were prepared immediately prior to treatment. Cells from different cell lines were treated with various concentrations, and the optimal effective dosage and durations of treatment were determined:

a. K9 cells were treated with 100, 200, 500 and 1,000 ug/ml for up to 48 hr with media renewed every 24 hr period;

b. BRL cells were treated with 10, 20, 50, 100 and 200 ug/ml for up to 24 hr;

c. PTK_2 cells were treated with 100, 150 ug/ml for 24 to 72 hr.

The duration of treatment varied from 3 to 72 hr with media renewed every 24 hr and the control samples incubated in media with 1% DMSO. A recovery study was performed when the PFDA media was replaced, upon 24 hr incubation, with inert media which was also renewed every 24 hr. Measurements were taken at membrane level over the cytoplasmic region where it was relatively free of granules and pigments. In addition, membrane measurements at cytoplasmic and nuclear regions and for confluent and non-confluent cultures were also made.

RESULTS

Initial experiments involved determining a non-lethal level of PFDA exposure for the three cell lines being tested. Exposure of the cells to concentrations of 10 to 1,000 ug/ml for for 3-72 hr revealed cell death (as evidenced by cytoplasmic blebbing, vacuolation, and nuclear pycnosis) for the three cell lines as follows: BRL, 80% mortality at 200 ug/ml for 3 hr; K9, 100% mortality at 200 ug/ml for 48 hr; PTK_2, 50% mortality at 200 ug/ml for 24 hr. Thus it appears that the cell lines have a differential sensitivity to PFDA.

Studies on membrane fluidity were conducted with concentrations of exposure below the lethal level. A dose dependent relationship was observed with respect to membrane fluidity (Figure 1). In Figure 1, it can be seen that increasing concentrations of PFDA result in a progressive increase in membrane fluidity for the K9 and BRL cells. No effect is observed on the PTK_2 cells. Furthermore, there is a differential sensitivity between the

two responding cell lines: BRL cells appear to be more sensitive than the K9 cells. The BRL cells have a threefold increase in fluidity at 100 ug/ml of PFDA whereas the K9 cells require 200 ug/ml to exhibit a threefold increase in fluidity.

Studies also were conducted to determine if the PFDA fluidity effect either was permanent or disappeared over time. Cells were exposed to PFDA at different concentrations for 24 hr periods, washed, placed in fresh culture medium, and fluidity measurements made over a 24 to 72 hr time period (Figures 2-3). It is evident that the PFDA-induced increase in membrane fluidity returns to the control (untreated) levels by 48 hr following removal of the PFDA.

Since previous studies with hydrazine (5) demonstrated that effects on membrane fluidity also resulted in a morphological effect on the cell surface, scanning electron microscopy was conducted on PFDA-treated cells. No differences between treated and untreated cells were observed with respect to membrane morphology (microvilli or surface morphology).

In an effort to determine if the variation between measurements was due to factors inherent to the FRAP technique or possibly to parameters surrounding the cells, a series of studies was conducted to examine membrane fluidity in confluent versus non-confluent cells and over the nucleus versus over the non-nuclear portion of the cytoplasm. It is evident from these data that the fluidity in the cell membrane that is over the nucleus is consistently higher than in the cell membrane over the cytoplasm (Figure 4). However, there does not appear to be a significant difference between confluent and non-confluent cell populations (data not shown).

DISCUSSION

The data presented demonstrates that PFDA does affect the fluidity of the cell membrane. This effect is dose dependent within the range of 50-200 ug/ml. Furthermore, the sensitivity of various cell lines to the PFDA is different. The PTK_2 kidney cell line shows little sensitivity to PFDA whereas the two liver cell lines demonstrate a marked response. This may reflect a target-tissue specificity of the PFDA for liver. Since the liver has already been demonstrated to be a primary target site for PFDA toxicity (1) in vivo, it is possible that this specificity is maintained in the in vitro situation. Both the BRL and K9 cells maintain the production of some hepatic enzymes in culture.

The effect of PFDA on the fluidity of the cell membrane appears to be transient. Within 24-48 hr of its removal, the fluidity returns to control levels. This would suggest that the nature of the PFDA-induced increase in membrane motility is not the result of a major structural or biochemical insult. The lack of any observable morphological alteration by scanning electron microscopy would support this conclusion. However, more sensitive measurements of the cell surface, such as the measurement of ionic gradients in electrically active neuronal or myocardial cells, could address this point.

With respect to the large variability between measurements in different cells of the same or different type, and even the same cell type on different days, little can be said. There does not appear to be any difference between the variability due to cell confluency. Though there is a difference between the membrane fluidity over the nucleus versus the non-nuclear region of the cytoplasm, this was probably not a factor in these

experiments since the great majority of FRAP measurements were made over the non-nuclear portion of the cytoplasm anyway.

In summary, this study demonstrates that the compound PFDA does directly effect the cell membrane by causing a transient increase in membrane fluidity. The response is observed in two liver cell lines and not in a kidney line. The two liver lines exhibit different levels of sensitivity. Finally, the method of FRAP appears to be a sensitive way to uncover subtle effects of toxic agents on the cell membrane and may ultimately be a valuable method not only to screen compounds but also to elucidate mechanisms of action.

ACKNOWLEDGMENT

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FIGURE LEGENDS

Fig. 1. Membrane fluidity following 24 hr of PFDA treatment at concentrations indicated. Cells were treated with PFDA for 24 hr, washed, and FRAP measurements were made. For K9 cells, two separate experiments, (a) and (b), are indicated. All means contained at least 10 measurements from 10 different cells.

Fig. 2. Recovery of fluidity in K9 cells. The time periods indicate the time in recovery (non-PFDA) medium following PFDA treatment with doses indicated. By 48 hr, membrane fluidity had returned to control levels.

Fig. 3. Recovery of fludity in BRL cells.

Fig. 4. Fluidity measurements in the cell membrane over the nucleus versus over the non-nuclear portion of the cytoplasm. Times represent measurements made on the same cultures at two time intervals.

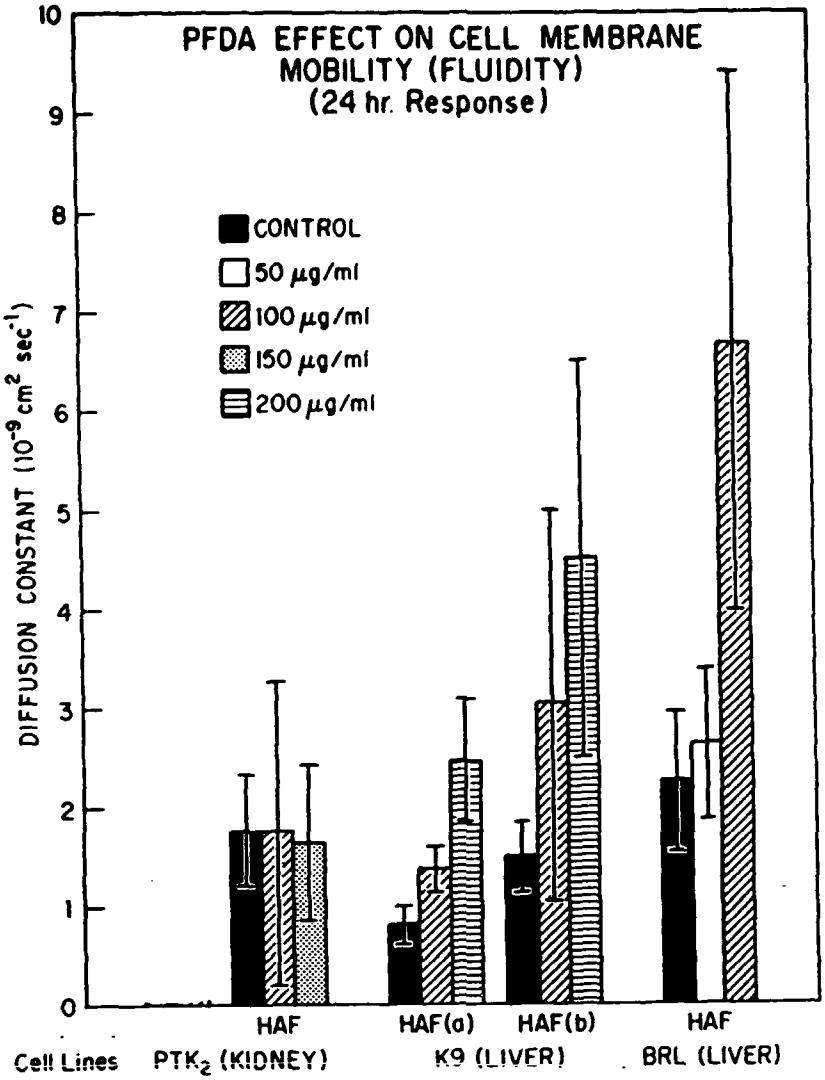
PFDA EFFECT ON CELL MEMBRANE
MOBILITY (FLUIDITY)
(24 hr. Response)
CONTROL
50 μg/ml
100 μg/ml
150 μg/ml
200 μg/ml
DIFFUSION CONSTANT (10^{-9} cm^2 sec^{-1})
0
1
2
3
4
5
6
7
8
9
10
HAF
HAF(a)
HAF(b)
HAF
Cell Lines
PTK$_2$ (KIDNEY)
K9 (LIVER)
BRL (LIVER)

Fig 1.

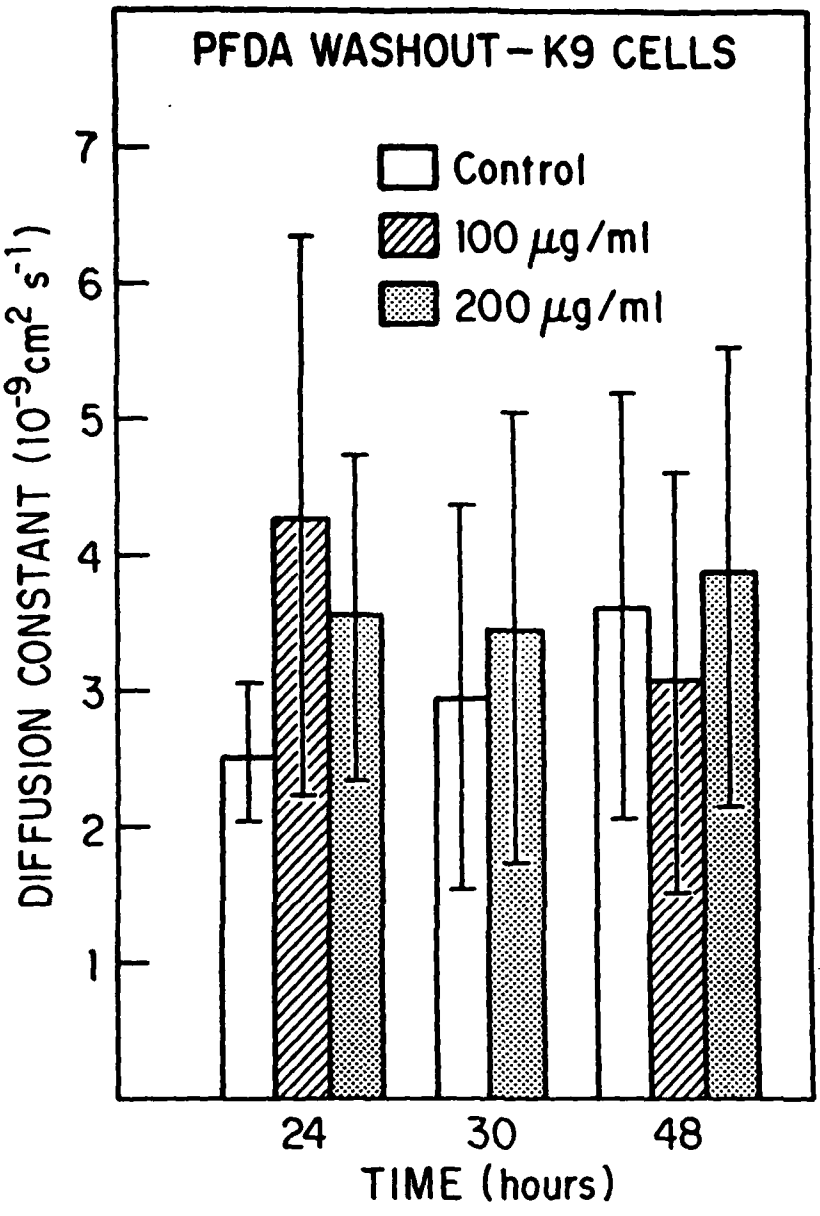
PFDA WASHOUT – K9 CELLS
Control
100 μg/ml
200 μg/ml
DIFFUSION CONSTANT (10^{-9}cm^2 s^{-1})
1
2
3
4
5
6
7
24
30
48
TIME (hours)

Fig. 2

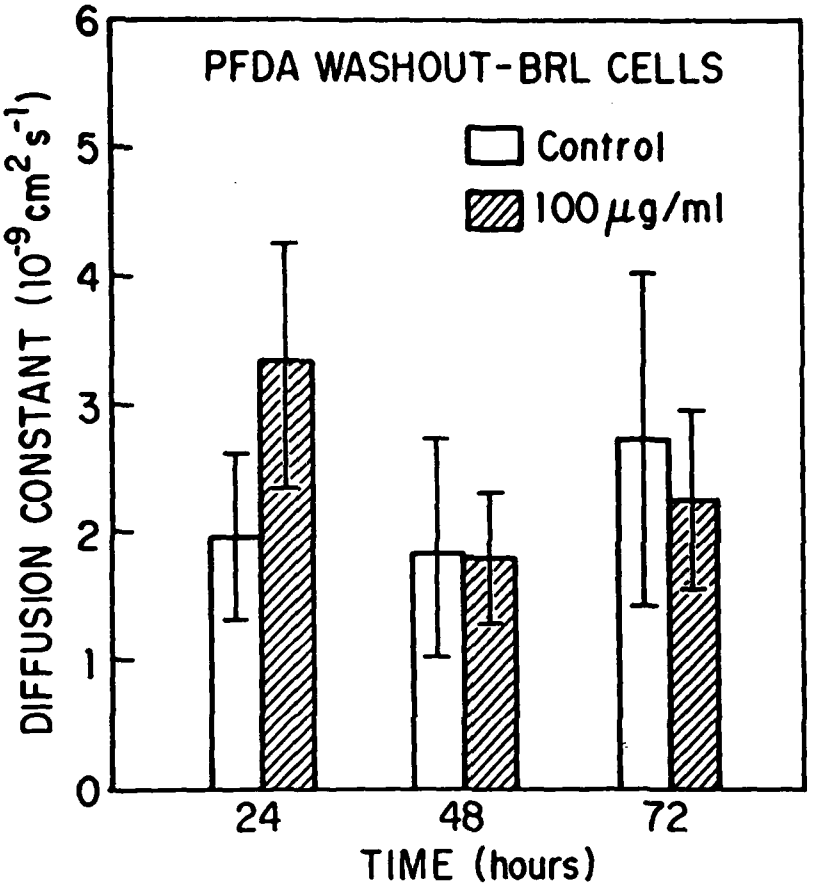
PFDA WASHOUT-BRL CELLS
Control
100 μg/ml
DIFFUSION CONSTANT (10^{-9} cm^2 s^{-1})
0
1
2
3
4
5
6
24
48
72
TIME (hours)

Fig. 3

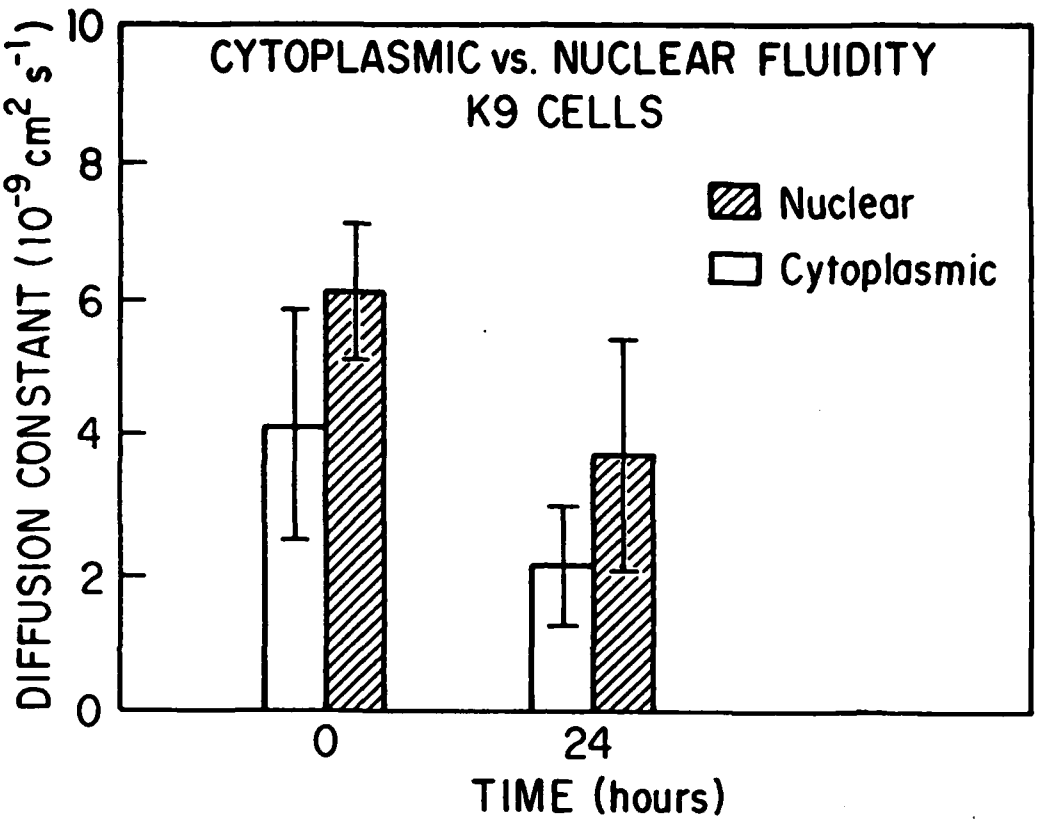
CYTOPLASMIC vs. NUCLEAR FLUIDITY
K9 CELLS
Nuclear
Cytoplasmic
DIFFUSION CONSTANT (10^{-9} cm^2 s^{-1})
10
8
6
4
2
0
0
24
TIME (hours)

Fig. 4

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